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(12) UK Patent Application (19) GB (11) 2 217 335 (13) A

(43) Date of A publication 25.10.1989

(21) Application No 8809576.5

(22) Date of filing 22.04.1988

(51) INT CL[4]
C08J 9/40

(52) UK CL (Edition J)
C3L LJX L180
U1S S1332 S1337

(56) Documents cited
JP-60256399
Biomaterials 1986, Vol.7, No 2, pages 121-125.

(58) Field of search
UK CL (Edition J) C3L LJC LJX
Online : Biosis, Chemical Abstracts, Derwent WPIL

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(54) Compositions for isolation and immobilisation of C-reactive protein in body liquids

(57) A diagnostic composition comprise phosphoryl choline (PC) residues and/or aminoethyl dihydrogen phosphate (AEDP) residues immobilised on a solid phase for quantitative and/or qualitative detection of C-reactive protein in test samples. The linkage between the PC or AEDP residues and the solid phase may be via a molecule different from the molecules of PC or AEDP or of the solid phase, or may be a direct linkage. The solid phase may be porous membrane of nitro-cellulose, polyamide or polyvinylidene difluoride, or polymeric surfaces e.g. of polystyrene or acrylic polymer.

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COMPOSITIONS FOR ISOLATION AND IMMOBILISATION OF C-REACTIVE PROTEIN IN BODY LIQUIDS

This invention relates to the isolation and immobilisation of C-reactive protein, which is an acute phase protein body liquids.

C-reactive protein (herein called CRP) was first reported in 1930 as a human serum protein that binds to the C-polysaccharide of the pneumococcus cell membrane (Tillet W.S. and Francis T: "Seriological reaction in pneumonia with a non-protein somatic fraction of pneumococcus", J. Exp. Med. 52: 561-571, 1930). Later it was demonstrated that C-reactive protein binds to phosphoryl choline residues and to aminoethyldihydrogen phosphate residues of this fraction, and these bindings have been utilized for the purification of C-reactive protein from serum. (Volanakais J.E. & al: J. Immunol. Methods 23, 285-295, 1978, and Potent M. & al: FEBS Lett. 88, 172-175, 1978).

Later it was discovered that CRP concentrations rise dramatically during pneumococcus infections and several forms of infectious inflammations (Abernathy TJ and Avery OP: "The occurrence during acute infections of a protein not normally present in the blood", J. Exp. Med. 73: 173-182, 1941). This non-specific response to bodily injury was classified as a part of the "acute-phase response", in which changes in the concentrations of serum proteins parallel the course of inflammation or tissue injury. Although their exact roles remain unclear, CRP and the other acute-phase proteins function as mediators, inhibitors or participants in the process of inflammation. CRP is a trace constituent in blood, where serum levels in healthy adults normally remain below 5 mg/l. The serum concentration of CRP rises rapidly after onset of infections, inflammations and tissue injury, and falls rapidly to normal levels as healing or recovery occurs (Whicher JT, Bell AM and Southall PJ: "Inflammation: Measurement in clinical management", Diagnostic Med. 81:62, 1981). As a clinical tool CRP quantitation appears to be more useful than the standard indices of inflammation, such as fever, erythrocyte sedimentation rate and leucocyte count. The magnitude of the incr ase from normal to acute inflammatory

concentration also contributes to the utility of CRP in monitoring disease activity (Pepys MB: "C-reactive-protein 50 years on; Lancet 1: 653-657, 1981).

The CRP molecule consists of 5 identical subunits held together by non-covalent bonds (Pepys MB: see above). Several methods for CRP detection and quantification are available, including immunoprecipitation (Andersen HC and McCarty M: "Determination of C-reactive protein in blood as measure of the activity of the disease process in acute rheumatic fever", Am. J. Med. 8: 445-445, 1950, and Wadsworth C: "A rapid spot immuno-precipitate assay method applied to quantitating C-reactive protein in paediatric sera". Scand. J. Immunol. 6: 1263-1272, 1977); latex agglutination (Singer JM, Potz CM, Pader E and Elster SK: "The latex agglutination test". Am J. Clin. Pathol 28: 611-617, 1957); radial immunodiffusion (Nilsson LA: "Comparative testing of precipitation methods for quantitation of C-reactive protein in blood serum". Acta Pathol. Microbiol. Scand. 73: 129-144, 1968); electroimmunodiffusion (Gil CW, Fisher CL, and Holleman CL: "A rapid method for protein quantitiation by electroimmunodiffusion", Clin. Chem. 17: 501-504, 1971); radioimmunoassay (Claus DR, Osman AP, and Gewurz H: "Radioimmunlassay of human C-reactive protein and labels in normal sera". J. Lab. Clin. Med. 87: 127-128, 1976 and Shine B, DeBeer FC, and Pepys MD: "Solid phase radioimmunoassay for human C-reactive protein". Clin. Chem. Act 117: 13-23, 1981); nephelometry (Deaton CD, Maxwell KW, Smith RF and Crevelling RL: "Use of laser nephelometry in the measurement of serum proteins". Clin. Chem. 22: 1465-1471, 1976 and Gil CW, Bush WS, Burleigh WM, and Fisher CL: "An evaluation of a C-reactive protein assay using a rate immunonephelometric procedure". AJCP 75: 50-55, 1981); fluoroimmunoassay (Anne L, Eimstad W, Bellet N, and Fisher C: "Development of homogeneous fluorescent rate immunoassay for C-reactive protein". Clin. Chem. 27: 1075, 1981 and Ullman F, Schwarzberg M and Rubensten KE: "Fluorescent excitation transfer immunoassay: A general method for the determination of antigens", J. Biol. Chem. 251: 4172-4178, 1976); and enzyme-labelled immunoassay (Gibbons I, Skiold C, Rowley GL and Ullman EF: "Homogeneous enzyme immunoassay for proteins employing beta-galactosidase". Anal. Biochem. 102: 167-170, 1981).

CRP is among th plasma proteins known to have th most prominent concentration difference between the normal plasma concentration and the plasma concentration in acute inflammatory and infectious-phase reactions. Since the increase in blood concentration of CRP is a very rapid response to infections and tissue damage, its determination is especially valuable in acute medical situations. A large number of acute medical incidents take place in general medical practice far from advanced medical laboratories. Most of the known methods for the detection and/or quantification of increased CRP utilize the laboratory equipment like spectrophotometers, radioactivity counters and nephelometric equipment, and a time-consuming isolation of serum or plasma from whole blood is necessary. Simple qualitative latex agglutination techniques do not utilize advanced equipment, but isolation of serum and/or plasma is necessary. A fast and simple method to detect and/or quantify acute-phase response of blood proteins in whole blood is thus needed.

All methods used in clinical practice for CRP in plasma or serum utilize immunoglobulins with specific affinity for these proteins. Such immunoglobulins may be of polyclonal or monoclonal origin. Polyclonal antibodies with specific affinity for CRP are obtained by immunization of animals with purified antigens. Monoclonal antibodies reactive to the said antigen may be obtained by fusion of spleen cells from animals immunized with antigen or fragments thereof with suitable cancer cell lines (Køhler and Milstein. "Continuous cultures of fused cells secreting antibodies of predefined specificity", Nature 286, 495-497, 1975). In vivo immunization techniques may also be applied. Several of the immunological techniques utilize modified antibodies, i.e. antibodies conjugated to enzymes or fluorescent residues or radioactive labels. Such modifications and conjugations may be obtained by numerous different known methods, as described by O'Sullivan MJ, Bridges JW and Marks V: "Enzyme immunoassay: A review", Annals of Clinical Biochemistry, 1979, 16, 221-240.

The present invention provides a diagnostic composition comprising phosphoryl choline residues and/or aminoethyl dihydrogen phosphate residues immobilised on a solid phase for quantitative and/or qualitative detection of C-reactive protein in test samples. Thus, the composition of the inv ntion has immobilised ligands for the in vitro quantitative and/or qualitative assay

of CRP in a test sample, usually fr m patients. The immobilised ligands are capable of binding CRP in presence of calcium (Ca^{++}) ions.

The supports chosen for these ligands are exemplified by different solid substances depending upon the design of the assay. A porous membrane or a polymeric surface is the preferred support for the C-reactive protein binding substances.

The present composition is used for designing in vitro diagnostics for a rapid assay of CRP both in veterinarian and in human medicine, either qualitatively or quantitatively.

The CRP-molecule consists of five identical subunits non-covalently associated with each other in a disc-like configuration. Each subunit of CRP has the ability to bind to both the phosphate and to the amino group of the ligands, creating a strong binding force. The dependency upon the Ca^{++} ions is not fully delineated, but it has been shown that addition of Ca-binding substances as EDTA (ethylenediamine tetra-acetic-acid) prevents and breaks the binding. Since CRP contains five identical subunits, one CRP-molecule has the ability to bind several PC or AEDP residues. In this way the principle for tagging CRP molecules to the immobilised PC and/or AEDP residues may be used for binding a second signal molecule to the CRP for quantification. Alternatively, the signal substance may be labelled with antibodies to the CRP molecule, thus using a different functional group for isolation and for detection of CRP.

The binding of the ligands is either directly to the solid support or through a linkage consisting of different chemical substances ranging from short bifunctional linkage substances up to large proteins. The use of a porous membrane as a solid support is used for the design of a dipstick test. Porous materials of nitro-cellulose, polyamide and polyvinylidene-difluoride are used.

Preactivation of these filter membranes to make them reactive to nucleophilic groups creates supports easily made CRP-reactive by exposure to substances like aminophenyl-phosphoryl-choline (APPC) and amino-alkyl-AEDP. These substances contain nucleophilic amino-groups which react with the membrane, forming covalent linkages between PC or AEDP residues and the membrane.

Alternatively, membranes containing functional groups as OH, amino, or carboxy at the surface could be used. In this situation, CRP-binding

substances such as APPC or amino-alkyl-AEDP are c valently linked to the surface by the use of bifunctional linkage substances. These reagents bind on the one hand to the amino-group of APPC or amino-alkyl-AEDP and on the other hand to an OH, amino or carboxy group of the membrane. Examples of such agents are bifunctional N-hydroxy-succinimide esters as bis(sulphosuccinimidyl)-suberate, which binds to amino-groups at both ends. Carbodiimides such as ethyl(dimethylaminopropyl)carbodiimide bind to amino-groups at one end and carboxy groups at the other, thus forming a peptide linkage.

Another approach is to immobilise proteins or peptides already made CRP-binding by first binding PC or AEDP residues to the protein. The immobilisation of such substances to the solid membrane may be performed by techniques described above.

By using polymeric surfaces such as polystyrene or polyacryl, it is possible to immobilise CRP-binding ligands such as PC or AEDP basically after the same principles as described for the porous membrane. By making the surface reactive to e.g. nucleophilic groups or introducing functional groups such as OH, amino or carboxy groups, a chemical linkage is made by the same substances as indicated above. By using hydrophobic surfaces, it is possible to immobilise proteins already having PC or AEDP substituents simply by adsorption of the protein on the surface.

The CRP-binding porous membrane may be designed to fit into a dip-stick test, where addition of a test sample containing CRP in the presence of Ca^{++} ions, will give a CRP-containing dip-stick after removal of other substances by washing. This bound CRP may be quantitative or qualitative, detected by a second CRP-binding signal substance. Preferably this may be an enzyme capable of transforming a substrate to a coloured insoluble product. Thus the dipstick may be read by the eye or a simple spectrophotometer/reflectometer.

Coloured colloids of gold/silver that have been made CRP-binding will give a coloured surface depending in intensity upon the amount of CRP isolated. This intensity is measurable by the eye or by simple equipment.

CRP-binding ligands linked to polymeric surfaces may be designed as cuvettes, tubes or 96-well microtitre plates. After addition of a sample c ntaining CRP and Ca^{++} the bound CRP may be determined by adding a second CRP-binding molecule, which will give a measurable signal depending

upon the nature f the substance. Enzymes labelled with PC or AEDP residues, or antibodies to CRP are used to give c loured solution after addition of substrate. The change in light absorbance will be in direct proportion to the bound CRP. Alternatively, a fluorescent agent and measurement of fluorescence after removal of not-bound substance will be used for quantification of CRP isolated from a test sample.

Scintillation counting for CRP measurements is used when a radioactive substance bound to a CRP-binding molecule are introduced to the isolated CRP.

CLAIMS

1. A diagnostic composition comprising phosphoryl choline residues and/or aminoethyl dihydrogen phosphate residues immobilised on a solid phase for quantitative and/or qualitative detection of C-reactive protein in test samples.

2. A composition according to Claim 1 in which the linkage between the phosphoryl choline and/or aminoethyl dihydrogen phosphate residues and the solid phase consists of a molecule different from the phosphoryl choline and/or aminoethyl dihydrogen phosphate residues and different from the molecules constituting the solid phase.

3. A composition according to Claim 1 or 2 in which the phosphoryl choline and/or aminoethyl dihydrogen phosphate residues are present in a polymeric form, with or without other molecules between the repeating monomeric units of phosphoryl choline and/or aminoethyl dihydrogen phosphate residues.

4. A compound according to any one of Claims 1 to 3 in which the solid phase is a porous membrane or a polymeric surface.

5. The use of a composition according to Claim 1, 2 or 3 for quantitative and/or qualitative detection of C-reactive protein in a test sample.

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